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Study of an aquifer contaminated by ethyl *tert*-butyl ether (ETBE): Site characterization and on-site bioremediation

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ABSTRACT

Ethyl *tert*-butyl ether (ETBE) was detected at high concentration (300 mg L^{-1}) in the groundwater below a gas-station. No significant carbon neither hydrogen isotopic fractionation of ETBE was detected along the plume. ETBE and BTEX biodegradation capacities of the indigenous microflora Pz1-ETBE and of a culture (MC-IFP) composed of *Rhodococcus wratislaviensis* IFP 2016, *Rhodococcus aetherivorans* IFP 2017 and *Aquincola tertiaricarbonis* IFP 2003 showed that ETBE and BTEX degradation rates were in the same range (ETBE: 0.91 and 0.83 mg L^{-1} h^{-1} and BTEX: 0.64 and 0.82 mg L^{-1} h^{-1}, respectively) but *tert*-butanol (TBA) accumulated transiently at a high level using Pz1-ETBE (74 mg L^{-1}). An on-site pilot plant (2 m^3) filled with polluted groundwater and inoculated by MC-IFP, successfully degraded four successive additions of ETBE and gasoline. However, an insignificant ETBE isotopic fractionation was also accompanying this decrease which suggested the involvement of low fractionating-strains using EthB enzymes, but required of additional proofs. The *ethB* gene encoding a cytochrome P450 involved in ETBE biodegradation (present in *R. aetherivorans* IFP 2017) was monitored by quantitative real-time polymerase chain reaction (q-PCR) on DNA extracted from water sampled in the pilot plant which yield up to 5×10^6 copies of *ethB* gene per L^{-1}.

1. Introduction

Fossil fuels such as gasoline are frequently found contaminants of groundwater [1]. Fuel oxygenates, methyl *tert*-butyl ether (MTBE) or ethyl *tert*-butyl ether (ETBE), are added to gasoline to replace tetra alkyl-lead because of their high octane index and more complete combustion. MTBE (added at 15%, v/v), was used during 80s–90s. Since 2003, in Europe, directives (Renewable Energy Directives 2003/30/EC and 2009/28/EC) have been adopted to promote the use of biofuels through tax incentives. European MTBE producers converted their plants to produce ETBE using bioethanol for synthesis. Consequently, ETBE is increasingly replacing MTBE in Europe and is the main way of bioethanol utilization with ETBE concentration up to 22% (v/v). MTBE and ETBE have a high water solubility (40 and 10 g L^{-1}, respectively) and a poor biodegradability. Numerous studies have shown the ubiquity of MTBE in groundwater [2–6]. MTBE persistence in water has raised much concern and this compound was considered as a possible human carcinogen by US EPA [7]. The environmental impact of ETBE was not estimated before its utilization in Europe where the level of ETBE contamination in groundwater is not known. ETBE properties similar to those of MTBE should lead to a similar environmental behavior [8]. Actually, recent reports of groundwater contamination in France: in Vaucluse district, (http://www.leparisien.fr/societe/alerte-au-carburant-dans-des-bouteilles-d-oasis-18-10-2008-280533.php) and in the city of Bordeaux (http://www.laviedesreseaux.fr/Informez-vous/Actualite/Eau-polluee-a-Bordeaux-l-ETBE-mis-en-cause) highlighted the possibility of ETBE contamination.

The European Commission has completed risk assessment on ETBE concluding that there was no health concern with it "in the

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case of non-repeated swallowing, contact with the skin and inhalation" but nothing is known about the possible risks following a long-lasting exposition to ETBE present in polluted aquifers. Moreover, ETBE has very low odor and taste thresholds in water (1 and 2 $\mu g\,L^{-1}$, respectively) rendering water undrinkable even at very low concentrations [9]. EU legislation in preparation should propose a limit for ETBE in water (20 $\mu g\,L^{-1}$). Nevertheless, there is no recommendation in the European countries utilizing ETBE for its detection when aquifers are contaminated by gasoline.

When an aquifer is polluted with oxygenates, the physico-chemical decontamination processes (pump-and-treat, ...) are not efficient [10] and biological processes (biobarrier, ...) could be cost-effective to treat the pollution [11]. Few microorganisms were able to grow on ETBE [12–14] and a cluster of *eth* genes encoding a cytochrome P450 monooxygenase system involved in the first step of the ETBE biodegradation pathway was characterized in *Rhodococcus ruber* IFP 2001 [15]. The *ethB* gene was shown specifically induced by ETBE [16]. The presence of the *ethB* gene was also detected in *Rhodococcus aetherivorans* IFP 2017 growing on ETBE [17]. These ETBE-degrading strains accumulated *tert*-butanol (TBA).

Recently, compound-specific stable isotope analysis (CSIA) has become an important tool for monitoring biodegradation of contaminants in environment [18,19], for characterizing the initial reaction mechanisms and for identifying degradation pathways [20]. The isotopic fractionation of ETBE by *R. ruber* IFP 2001 expressing the EthB cytochrome P-450 is leading to a low carbon and hydrogen enrichment factor [21].

Complementarily, DNA-based molecular tools such as quantitative real-time PCR (qPCR) have become important to prove the biodegradation of pollutants in contaminated sites. The increase in microorganisms with specific biodegradation capacities by measuring the copies of 16S rRNA genes was carried out at a site contaminated by MTBE by showing an increase in *Methylibium petroleiphilum* PM1-like 16S rRNA copies [22]. Nevertheless, it is generally considered preferable to assess the presence and number increase of catabolic genes specifically involved in the pollutant biodegradation [23–25].

In the framework of the TISATIE project granted by AXELERA pole (http://www.business.greaterlyon.com/axelera-chemical-environmental-lyon-competitiveness-cluster.82.0.html?&L=1), we studied a leaking gas station site (Rhône district, France) showing the contamination of the underneath groundwater by high ETBE concentrations. We report here on (i) the characterization of the ETBE-polluted site using hydrogeological tools, (ii) the determination of the best way for treating the pollution either using biostimulation or bioaugmentation with Pz1-ETBE consortium enriched from the indigenous microflorae or with MC-IFP culture, composed of *Rhodococcus wratislaviensis* IFP 2016, *R. aetherivorans* IFP 2017 and *Aquincola tertiaricarbonis* IFP 2003 (CIP I-2052), with large capacities towards gasoline compounds [17,26], (iii) the use of MC-IFP for bioaugmentation in an on-site pilot plant (2 m^3) and (iv) the investigation of two ways for assessing the ETBE biodegradation effectiveness under *in situ* conditions using carbon and hydrogen isotopic fractionation measurements and determination of the abundance of a specific gene (*ethB*) through qPCR.

To the best of our knowledge, this is the first characterization of an ETBE-contaminated groundwater and the first attempt for bioremediation at a site where ETBE is the main pollutant.

2. Materials and methods

2.1. Geological characterization

The site is located in an active gas-station equipped with 40 piezometers. A geological characterization and the hydrodynamic transport of the soluble pollutants in the aquifer were determined. Stratigraphic cross section maps allowed the lithologic characterization.

2.2. Hydrodynamic characterization

The hydrodynamic behavior of the water fluxes in piezometers was studied to identify the hydraulic linkage between the alluvial layer and the perched water table. Aquifer productivity and soil permeability have been determined at different depths in various piezometers with (i) common slug test and (ii) conductimetric diagraphic test. Fluorescent tracers were used to estimate the dynamic flow rate of the aquifer.

2.3. Physico-chemical characterization of the pollutants

A site investigation has been performed in the hot spot and in the plume.

Water samples were regularly taken from the wells. Redox potential, conductivity, pH, dissolved O_2 and temperature were measured in the groundwater with on-site probes. Water samples were taken in gastight vessels containing H_2SO_4 for preservation before laboratory analyses and stored in the dark and at 4 °C before analysis.

The pollutants dissolved in water (ETBE, BTEX, MTBE and TBA) were analyzed as described below.

Dissolved O_2 was measured by a portable analyzer (Hach Lange, Germany).

2.4. Bioremediation study at lab scale

2.4.1. Strains and preservation

Stock cultures of Pz1-ETBE, obtained by enrichment of the indigenous Pz1-groundwater microflorae on ETBE (results not shown), of *R. wratislaviensis* IFP 2016, *R. aetherivorans* IFP 2017 [17,29] and *A. tertiaricarbonis* IFP 2003 (CIP I-2052) [26–28] were kept frozen at −80 °C in mineral medium (MM) [17] containing 20% glycerol (v/v).

2.4.2. Preparation of precultures

Pz1-ETBE was cultivated in 3-L flask closed with a stopper containing 400 mL MM. After inoculation with the consortium (25%, v/v), ETBE was added (200 $mg\,L^{-1}$). The flasks were incubated at 30 °C under agitation (120 rpm).

MC-IFP was composed of *R. wratislaviensis* IFP 2016, *R. aetherivorans* IFP 2017 and *A. tertiaricarbonis* IFP 2003. Each strain was grown at 30 °C for 72 h under agitation (120 rpm) in 150 mL Tripticase Soy (TS). The three cultures were centrifuged (8000 × *g* for 10 min) and washed with MM. MC-IFP was prepared by mixing the strains at equal part (based on biomass) and used as the inoculum ($O.D._{600\,nm} \sim 1$) in a 5-L Schott flask sealed with a stopper and containing 1 L MM. The carbon sources were ETBE (300 $mg\,L^{-1}$) and a mixture (equal part of each monoaromatic compound) of BTEX (100 $mg\,L^{-1}$) added as described above.

2.4.3. Biodegradation capacities determination under site conditions

- **Fixation of the cultures on perlite**: the potential of perlite (Carlo Erba, Italy) was previously studied [29]. Perlite was washed, dried and packed in 8-cm length columns (∼1.7 g perlite/column). When ETBE and BTEX have been degraded, the cultures Pz1-ETBE or MC-IFP were centrifuged (8000 × *g* for 10 min), washed with MM and re-suspended in 25 mL of MM. Each culture was percolated on a perlite column.
- **Cultures on contaminated groundwater**: the experiments were carried out in tightly closed 5-L Schott flasks with a side-arm.

The flasks contained 1 L of unfiltered groundwater from Pz10. Calculations were made after water content analysis so that the concentration of the main minerals, i.e. nitrogen, calcium, magnesium and iron would not be limiting. Thus, NH_4NO_3 was added at 100 mg L^{-1}, $FeSO_4$, $7H_2O$ at 1 mg L^{-1}. KH_2PO_4 at 1.40 g L^{-1} and K_2HPO_4 at 1.70 g L^{-1} were added before inoculation. The carbon sources were the contaminants present in Pz10-groundwater, i.e. ETBE (196 mg L^{-1}), BTEX (37 mg L^{-1}), MTBE (1.5 mg L^{-1}) and TBA (4.2 mg L^{-1}). Oxygen was not limiting at such substrate concentrations.

Biostimulation was carried out in two flasks containing Pz10-groundwater to determine if oxygen (Flask 2) or oxygen and minerals (Flask 3) were sufficient to promote biodegradation by the indigenous microflora. The control was mimicking the site conditions and consisted of a flask completely filled with groundwater (Flask 1).

Bioaugmentation using Pz1-ETBE or MC-IFP was carried out in two flasks filled with Pz10-groundwater in the presence of oxygen and of the salt solution as described above. Flasks were inoculated with perlite columns containing either Pz1-ETBE (Flask 4) or MC-IFP (Flask 5). The control of these experiments corresponds to a flask containing groundwater and a sterilized-perlite column (Flask 6).

The flasks were incubated under agitation (100 rpm) at 15 °C (groundwater temperature). Liquid samples were regularly taken for ETBE, BTEX, MTBE and TBA analyses. The gaseous phase was sampled using a gas-tight syringe for CO_2 measurement.

2.5. On-site pilot plant for biotreatment

2.5.1. Preculture

MC-IFP was chosen for site biotreatment. MC-IFP preculture was prepared on TS (see above). The cells of each strain were collected and inoculated in MM (5 L). ETBE and the BTEX mixture were regularly added (total concentration finally added: 3.37 g L^{-1} and 0.94 g L^{-1}, respectively). The preculture was regularly aerated to prevent O_2 limitation. After several sub-cultures of increasing volume, 55 L of MC-IFP were obtained. This preculture was used to inoculate 21 columns (1-m length, 10-cm diameter PVC tubes) filled with perlite.

2.5.2. Pilot plant-scale treatment

The 2-m^3 bioreactor (0.8 m × 1.6 m × 1.2 m) was filled with 1.5-m^3 groundwater pumped in parallel at Pz10, Pz18, Pz17 and P2 (gaseous phase = 0.5 m^3). Twenty-one columns containing perlite inoculated with MC-IFP culture were introduced in the bioreactor which was tightly closed to avoid volatilization. The pilot plant was conducted in a fed-batch mode without any temperature or pH regulation.

Four additions of ETBE and SP 98 gasoline were carried out. ETBE, BTEX and TBA concentrations were monitored by GC/FID. H_2O_2 was injected at a rate adjusted to the O_2 consumption rate (O_2 concentration $\geq$ 10 mg L^{-1}).

2.6. Analytical procedures

ETBE, BTEX, MTBE and TBA were analyzed in water samples (i) by a method combining headspace injector (HS)/GC/FID and the use of HydroCARB® software or (ii) by direct on-column GC/FID:

(i) For low concentrations, the analytes were quantified by flame ionization detection on a Varian gas chromatograph (Varian, France) equipped with a 0.2 mm × 50 m PONA capillary column with a 0.5-μm stationary phase composed of methylpolysiloxane (J&W Scientific, Chromoptic, Auxerre, France), using a two-step temperature gradient ranging from 35 °C to 114 °C at 1.1 °C min^{-1}, then from 114 °C to 310 °C at 1.7 °C min^{-1}. The injector was a static headspace injector (HS). Helium (0.8 mL min^{-1}) was the carrier gas. The detection limits under these conditions were 4.5 μg L^{-1} for MTBE and each BTEX, 3.7 μg L^{-1} for ETBE and 100 μg L^{-1} for TBA.

(ii) ETBE, MTBE and TBA were also quantified by flame ionization detection on a Varian 3300 gas chromatograph (Varian, France) [17].

Carbon dioxide was quantified by thermal conductivity detection (GC/TCD) as previously described [17].

2.7. Compound specific isotope analysis (CSIA)

Carbon and hydrogen isotope analyses were performed to determine if ETBE biodegradation was occurring along the plume and to elucidate the predominant reaction mechanism in microcosm studies with Pz1-ETBE and in the biotreatment pilot plant.

Stable isotope calculations, definition of parameters and analytical methodologies can be found in the Supplemental Data file.

2.7.1. ETBE isotopic signatures in the plume

Groundwater samples were taken in November 2009 from 12 wells (close to the source, along and downstream the plume) to compare their ETBE carbon and hydrogen isotopic signatures. Depending on the expected ETBE concentrations, the samples were preserved with 70 g of NaCl for around 200 mL sample in 240-mL serum bottles for manual HS injection or with 1% (w/w) $Na_3PO_4{\cdot}12H_2O$ in 1-L bottles for purge and trap (P&T) preconcentration step. The 240-mL bottles were closed with Teflon stoppers and aluminum caps, preheated at 60 °C and analyzed by manual HS [21], the P&T system being only connected to the instrument measuring carbon isotope composition.

2.7.2. Determination of ETBE isotopic enrichment factors by Pz1-ETBE

Potential variability of ETBE isotope fractionation by Pz1-ETBE was evaluated in resting cells experiments ($O.D._{700\,nm} \sim 0.1$) under different oxygen concentrations (oxic 21% vs. hypoxic <2% O_2 in the headspace) and incubation temperatures (12 °C vs. 30 °C). All the cultures were incubated in 240-mL serum bottles equipped with oxygen sensor spots (POF-PSt3, PreSens, Germany) and filled with 60 mL phosphate-buffered mineral medium (310 mg P L^{-1}, pH 7.5) and ETBE at 300 mg L^{-1}. Culture preparation, sampling and monitoring concentrations of ETBE, TBA and O_2 as well as carbon and hydrogen isotopic composition of residual ETBE were previously described for MTBE [30].

2.7.3. ETBE isotopic fractionation in the biotreatment pilot plant

For ETBE carbon and hydrogen isotopic analysis by P&T, 8-L water samples were taken in the pilot plant for a 240-h period during degradation of the fourth and last addition of ETBE and SP98 gasoline (beginning at 2300 h) in the biotreatment pilot and analyzed as described in the Supplemental Data file.

2.8. Quantitative real time PCR (qPCR)

DNA was extracted from pilot-plant samples (1 L) at different times using the UltraClean™ water DNA isolation kit (Mobio kit, Carlsbad, CA) according to the manufacturer instructions. Absolute quantification of *ethB* gene was performed using the Light Cycler® 480 Real-Time PCR System (Roche Diagnostics-Applied Science, Mannheim, Germany) using optical grade 96-well plates (final volume = 20 μL). SYBR Green reaction mixture contained 2 μL of template DNA, 200 nM of primers ethBfor and ethBrev [16] and

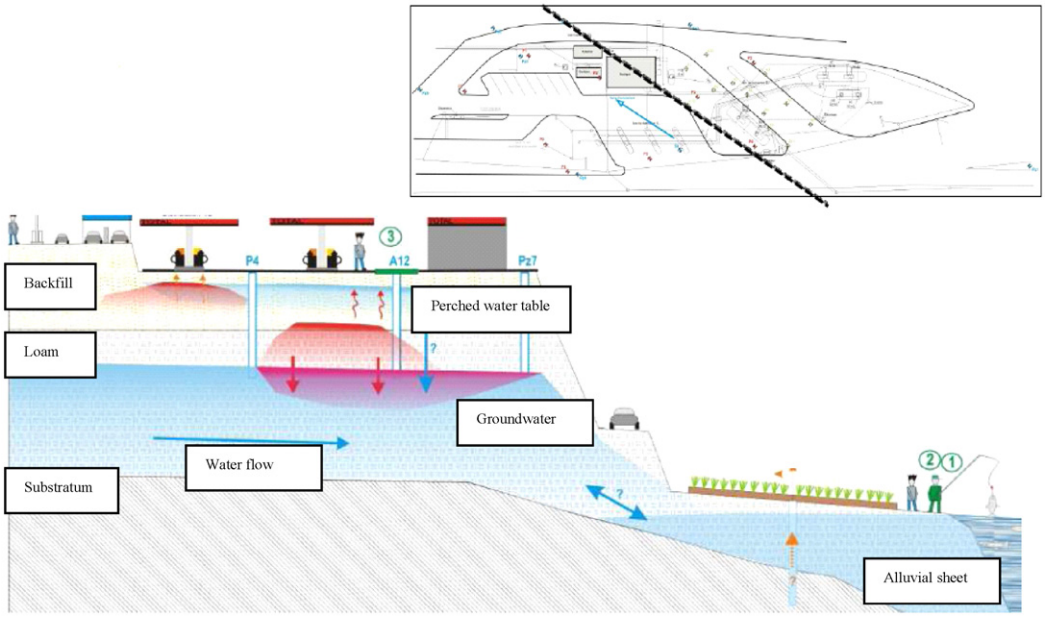

Fig. 1. Map of the site. Dots correspond to piezometers.

14 μL of Light Cycler® 480 SYBR Green I Master (Roche Diagnostics-Applied Science, Mannheim, Germany). The PCR program consisted of 10 min at 95 °C, followed by 50 cycles with 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. A dissociation curve was added to SYBR Green assays to check the specificity of the amplification.

Standard curves were generated by amplification of the serial ten-fold dilutions of purified PCR products (from 10^2 to 10^9 copy number L^{-1}). PCR products concentrations were measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and *ethB* gene copy number was calculated using the molar extinction coefficient and the Beer-Lambert equation. The copy numbers were calculated from the standard curves. *EthB* gene was not detected at concentration < 10^3 copies L^{-1}.

3. Results and discussion

3.1. Geology and hydrodynamic

We investigated the geology and the hydrodynamic network of the aquifer. Despite the complexity of flow summation, the widespread lithology was determined by conductimetric diagraphy and showed a high heterogeneity of soil: sandy silt clay (10^{-4}–10^{-5} m s^{-1}), black and grey silt (2×10^{-5} to 3×10^{-6} m s^{-1}), brown clay (10^{-5}–10^{-6} m s^{-1}), altered rocks and gneiss (10^{-6} m s^{-1} to 0). A conceptual scheme of the hydraulic functioning of the site was designed (Fig. 1). Two main active aquifers have been detected: one alluvial layer and a perched water table in the talus.

The fluctuations of the water table between the river and the piezometer network revealed a hydraulic barrier along the talus constituted by the foundation of an old buried canal. Despite this barrier, ETBE detection in the downstream area around Pz7, Pz14, Pz20 and Pz15 (alluvial aquifer) and the high hydraulic gradient suggested a flow from the talus to the alluvial aquifer which was confirmed by the presence of fluorescent tracers. This suggested that either the flow passes through a small fractured zone in the old canal foundation or that the overall permeability along the talus is very low and the flow very slow leading to very long tracer time responses.

3.2. Characterization of the pollution

3.2.1. Pollutant distribution in the plume

High ETBE (175 mg L^{-1}) but lower BTEX (10 mg L^{-1}) and MTBE (2.3 mg L^{-1}) concentrations were characterized in the aquifer by HS/GC/FID (January 2008). The main pollutant source was near the oil storage tank.

In the plume downstream the site (Fig. 2a and b), the most impacted piezometers (April 2010) were Pz10, Pz16, Pz17, Pz18, Pz19, A2, and P3 with a hot spot in Pz19 (301 mg L^{-1} of ETBE and 51 mg L^{-1} of BTEX).

As shown in Fig. 2a and b, the ETBE plume is larger than the BTEX's most probably due to its higher water solubility and lower biodegradability according to what was reported for MTBE plumes [31]. Moreover, due to the low log Kow for BTEX, these compounds are retained in the organic matter in soil contrarily to ETBE or MTBE.

3.2.2. Physico-chemical characterization of the aquifer

Since 2008, the analysis revealed mostly hypoxic conditions in downstream piezometers located between Pz9 and Pz13 (0.25–0.5 mg L^{-1} O_2). Higher dissolved oxygen values were detected upstream the site near Pz1 and Pz8 (1.5 and 2.6 mg L^{-1} O_2, respectively). In the hot spot, other electron acceptors (nitrate, sulfate) were totally consumed and sulfide or nitrite were detected (results not shown). Low pH values and a negative redox potential were also measured.

3.2.3. ETBE isotopic signatures in the plume

Despite the wide range of ETBE concentrations (from 120 mg L^{-1} to 64 μg L^{-1} along the plume in November 2009), no significant carbon ETBE isotopic fractionation was detected when comparing $\delta^{13}C$ values along the plume (average -27.3 ± 0.3‰). The difference of $\delta^{13}C$ values are similar to those obtained closer to the source and rarely exceeded the total analytical uncertainty ±0.5‰ [32]. The same was observed for hydrogen (mean $\delta^2H = -200 \pm 7$‰), although the HS technique was only able to analyze ETBE concentration >1 mg L^{-1}.

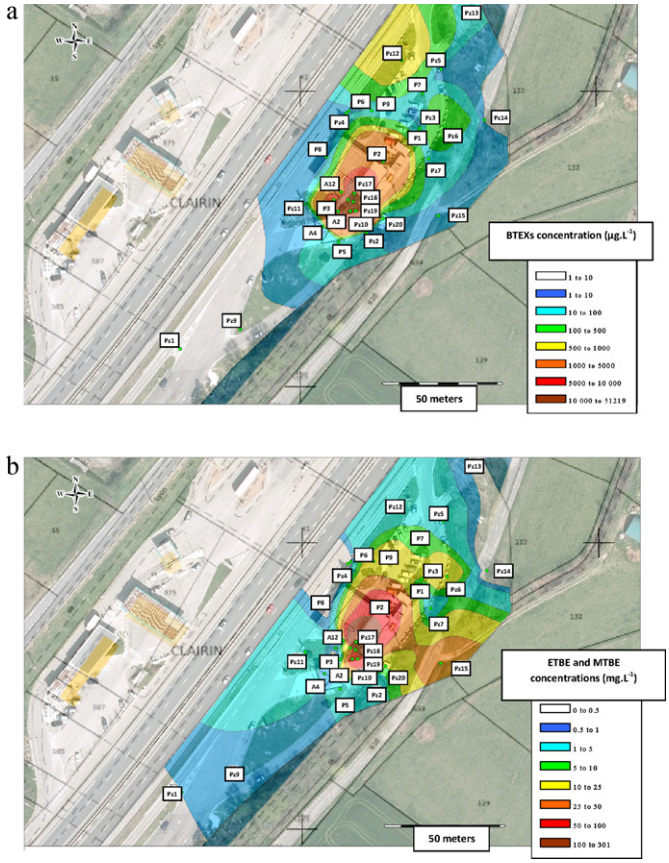

Fig. 2. BTEX (a) and ETBE (b) concentrations in the plume (April 2010).

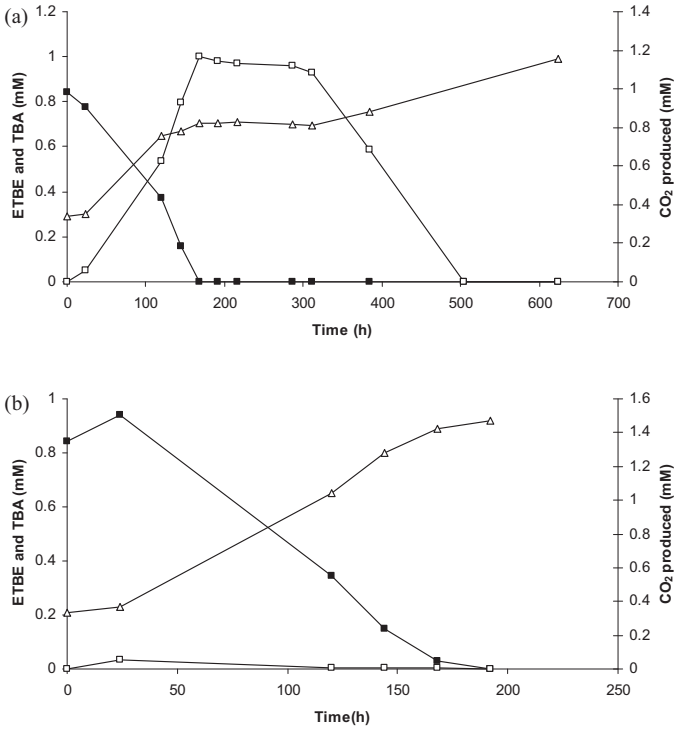

Fig. 3. Biodegradation of ETBE in groundwater using (a) Pz1-ETBE or (b) MC-IFP. ETBE (■-■) and TBA (□-□) were measured by GC/FID and CO_2 (▲-▲) by GC/TCD. ETBE and TBA are expressed as mM (calculated in the liquid phase). CO_2 produced is expressed as mM (calculated in the gaseous phase). Bioaugmentations by Pz1-ETBE and MC-IFP were carried out via perlite-columns previously inoculated.

3.3. Determination of the optimal conditions for site treatment

3.3.1. Biostimulation vs. bioaugmentation

We compared the biodegradation efficiency towards ETBE and BTEX present in the groundwater of the site, at 15 °C and under different laboratory conditions: (i) the biostimulation by oxygen addition (Flask 2), (ii) the biostimulation by oxygen and a salt solution addition (Flask 3), (iii) the bioaugmentation/biostimulation using Pz1-ETBE (Flask 4) or MC-IFP (Flask 5).

No biodegradation of ETBE was observed when biostimulation was carried out (Flasks 2 and 3) even after 500 h of incubation (Table 1), whereas total ETBE biodegradation to CO_2 and biomass was achieved in Flask 4 after 500 h of incubation (Fig. 3a and Table 1) or in Flask 5 after 200 h of incubation (Fig. 3b and Table 1). In the latter case, no significant TBA production ($\leq$2.6 mg L^{-1}) was measured whereas, in the former case, a significant amount of TBA ($\sim$75 mg L^{-1}) accumulated by Pz1-ETBE and was degraded in a second step.

Table 1
ETBE and BTEX biodegradation rates under different laboratory conditions.

Conditions of the assay	ETBE biodegradation rate (mg L^{-1} h^{-1})	BTEX biodegradation rate (mg L^{-1} h^{-1})
Site conditions[a]	0	0
Biostimulation-1[b]	0	0.04
Biostimulation-2[c]	0	0.02
Bioaugmentation (Pz1-ETBE consortium)[d]	0.91	0.64
Bioaugmentation (MC-IFP mixed culture)[e]	0.83	0.82
Control-sterile perlite	0	0.04

[a] Site conditions (Flask 1): a 5-L flask completely filled with groundwater (no oxygen available).
[b] Biostimulation (Flask 2): 5-L flask containing 1 L of groundwater (oxygen alone).
[c] Biostimulation (Flask 3): 5-L flask containing 1 L of groundwater (oxygen and salt addition).
[d] Bioaugmentation (Flask 4) 5-L flasks containing 1 L of groundwater (oxygen and salt addition) and a perlite-column inoculated with Pz1-ETBE.
[e] Bioaugmentation (Flask 5) 5-L flasks containing 1 L of groundwater (oxygen and salt addition) and a perlite-column inoculated with MC-IFP.

No ETBE biodegradation was observed in the controls (Flask 1 mimicking the site conditions and Flask 6 containing sterile perlite) of these experiments after 500 h (Table 1).

Our results clearly demonstrated that biostimulation alone was not efficient for ETBE remediation at the site, the ETBE biodegraders being probably too scarce in the groundwater. Few microbial species possess the enzymes capable to degrade the pollutant could explain the failure of biostimulation [33]. On the contrary, when bioaugmentation/biostimulation was carried out either with Pz1-ETBE (indigenous microflorae enriched on ETBE, results not shown) or with MC-IFP, ETBE was biodegraded. No adsorption of ETBE was observed on sterile perlite (Flask 6) and a similar result was previously obtained with MTBE [29]. The bioaugmentation using MC-IFP led to a more rapid ETBE biodegradation than using Pz1-ETBE and TBA was not produced at a high level in the former case. Accumulation of TBA was previously found using *Mycobacterium austroafricanum* IFP 2012 during MTBE biodegradation [34] and also using MTBE-degrading microcosms under site conditions [35]. The two-step pattern of CO_2 production corresponded to the ETBE biodegradation profile in the case of Pz1-ETBE (Fig. 4a) and was not observed using MC-IFP which confirmed that ETBE was degraded to CO_2 without TBA transient accumulation (Fig. 3b).

Different results were obtained with the BTEX. No degradation was observed in the control (Flask 1) but BTEX biodegradation was observed in all the other cases (Flasks 2, 3, 4 or 5) but at different rates (Table 1). This result is in agreement with the fact that BTEX

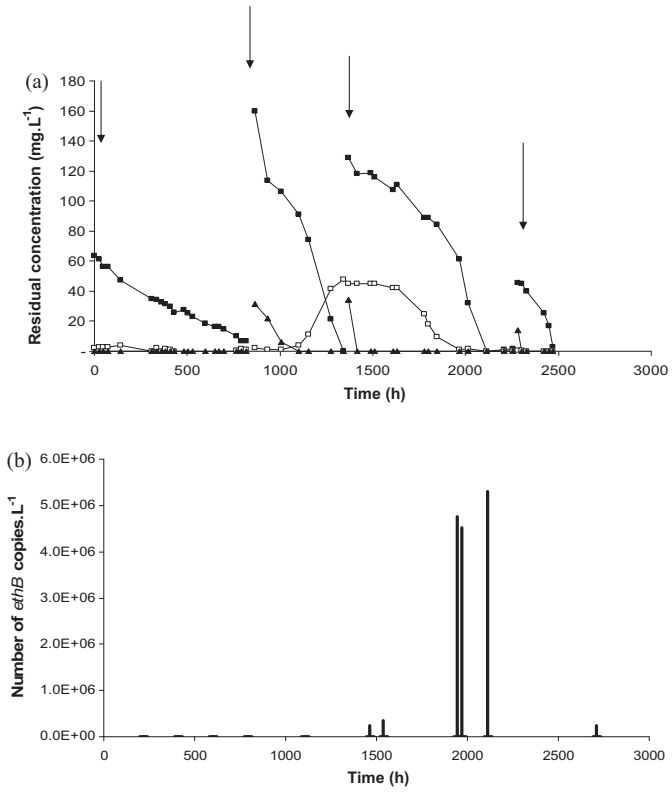

Fig. 4. Biotreatment in a pilot plant using MC-IFP. (a) Evolution of concentrations and (b) variations of the *ethB* gene abundance. (a) ETBE (■-■) and TBA (□-□) were measured by GC/FID and BTEX (▲-▲) by headspace GC/FID. (b) Each value corresponds to two independent measurements. Four additions of ETBE and SP98 gasoline were performed (arrows).

biodegraders are ubiquitous in the environment [35,36]. Both Pz1-ETBE and MC-IFP showed higher BTEX biodegradation capacities. In the case of MC-IFP, *R. wratislaviensis* IFP 2016, previously showed degradation capacities towards each of the six BTEX [17]. BTEX was also depleted in the presence of sterile perlite due to adsorption onto perlite (#15% of BTEX removal) since BTEX readily adsorbed on mineral supports [37].

Both Pz1-ETBE and MC-IFP were able to work in groundwater thus showing their capacity to undergo competition with indigenous microorganisms. Pz1-ETBE, itself composed of indigenous microorganisms, was not supposed to enter into competition with indigenous microorganisms [33]. Moreover, we fulfilled a condition considered as crucial in achieving bioaugmentation by protecting the microorganisms from adverse circumstances by a biofilm structure (i.e. fixation on perlite) [33]. van der Gast et al. [38] showed that the use of a well-defined consortium of previously selected strains (as MC-IFP) also led to higher performances by comparison with an undefined indigenous community (as Pz1-ETBE). Bioaugmentation was also proven efficient in the case of MTBE-contaminated aquifers when intrinsic microflora had no MTBE-biodegradation capacities [22,39].

Both cultures carried out ETBE and BTEX biodegradation at 15 °C which was the mean temperature of the aquifer.

3.3.2. Pz1-ETBE isotopic fractionation pattern

In the microcosms studies performed with a concentrate of cells of Pz1-ETBE, a very low carbon and almost non detectable hydrogen fractionation of ETBE was observed during biodegradation (Table 2). Pz1-ETBE was able to completely degrade ETBE under all tested conditions, except for the hypoxic and 12 °C which experiment was stopped after 18 days with an ETBE degradation of 85%. Curiously, TBA was practically not accumulated under hypoxic conditions (not detected at 30 °C and only up to 20 mg L^{-1} at 12 °C) whereas high concentrations were reached under oxic conditions. The ETBE carbon enrichment factors (ε_C from −0.4 to −0.7‰) obtained under different oxygen and temperature conditions did not vary significantly. Therefore, the ETBE degraders from Pz1-ETBE seem to follow the same enzymatic reaction mechanism under the different studied conditions. Comparing to previous studies, the obtained ε_C are similar to the ones observed for *A. tertiaricarbonis* L108 and *R. ruber* IFP 2001. In both strains, the initial monooxygenase reaction attacking the ethoxy group of ETBE is catalyzed by a cytochrome P450 (CYP249) encoded by the *ethB* gene which has been found to be highly conserved in other strains able to grow on ETBE [16]. The low fractionation pattern discarded any implication of the other studied strain, *Pseudonocardia tetrahydrofuranoxydans*, K1 ($\varepsilon_C = -1.7$ and $\varepsilon_H = -73$‰) attributed to the expression of a tetrahydrofuran monooxygenase.

3.4. Pilot plant for biotreatment

3.4.1. ETBE and BTEX biodegradation in the bioreactor

After inoculating MC-IFP (4th August 2010), four successive fed-batches (ETBE and SP98) were carried out. The evolution of ETBE and BTEX concentrations is shown in Fig. 4a. No MTBE was detected after filling the tank. The initial BTEX concentrations at each fed-batch never exceeded 35 mg L^{-1}. Total BTEX consumption was observed within 240 h at an average rate of 0.15 mg BTEX L^{-1} h^{-1}. Each new ETBE addition was followed by a lag phase corresponding most probably to a preferred assimilation of BTEX as previously reported in similar experiments [35]. At steady state, the average ETBE biodegradation rate during the last 3 substrate additions varied from 0.18 to 0.32 mg ETBE L^{-1} h^{-1}. TBA accumulation occurred transiently in the reactor (47 mg L^{-1}) and then was later mineralized. To increase the initial population of *A. tertiaricarbonis* IFP 2003 responsible for TBA assimilation [27] in the mixed culture MC-IFP could help limiting TBA accumulation.

Dissolved O_2 (H_2O_2 injection) never exceeded 10 mg L^{-1}. Average O_2 consumption rate during the first fed-batch reached 0.28 mg O_2 L^{-1} h^{-1} which was coherent with the ETBE theoretical oxygen demand (2.82 mg O_2 mg^{-1} ETBE) leading to a theoretical oxygen consumption rate of 0.26 mg O_2 L^{-1} h^{-1}. Since groundwater was used in the pilot plant, the possibility that indigenous microorganisms could also be involved in ETBE biodegradation process cannot be excluded even if we demonstrated (Table 1) that biostimulation alone did not allow ETBE biodegradation but only BTEX and at a lower rate than mentioned here above (0.04 vs. 0.15 mg L^{-1} h^{-1}).

3.4.2. ETBE isotopic profiles in the pilot plant

In spite of the decrease of ETBE concentration from 46 to 3 mg L^{-1} in the pilot plant after ten days of the last addition, no significant fractionation of the carbon and of the hydrogen was detected. At least for carbon a shift of 1‰ in the $\delta^{13}C$ value (from −22.6 to −21.6‰) suggests very low fractionating degraders in the pilot plant. This is consistent with the fact that *R. aetherivorans* IFP 2017, the ETBE degrader in MC-IFP, possess the P450 cytochrome (Table 2).

3.4.3. EthB gene abundance (qPCR) in the pilot plant

The abundance of the *ethB* gene encoding the P450 cytochrome and previously amplified from *R. aetherivorans* IFP 2017 [26] present in MC-IFP was monitored by qPCR on groundwater sampled in the pilot plant at different times. The results are shown in Fig. 4b.

The first determination carried out 216 h after the inoculation of the pilot plant with MC-IFP showed a low level of *ethB* copy

Table 2
Comparison of carbon and hydrogen enrichment factors (ε) and Λ values ($\Delta\delta^2H/\Delta\delta^{13}C$) for aerobic biodegradation of ETBE by consortium Pz1-ETBE at different incubation conditions and other pure strains reported in the literature. All the values have associated their ±95% confidence intervals (CI).

Culture	ε_C [‰]	±95% CI [‰]	R^2	B [%]	N	ε_H [‰]	±95% CI [‰]	R^2	B [%]	N	$\Lambda \pm 95\%$ CI	Reference
Pure aerobic cultures												
Aquincola tertiaricarbonis L108[a,b]	−0.67	0.04	0.93	98.9	69	−11.4	0.6	0.7	99.7	74	14 ± 1	[21]
Rhodococcus ruber IFP 2001[a]	−0.8	0.2	0.96	98	23	−10	2	0.8	98	23	10 ± 3	[21]
Pseudonocardia tetrahydrofuranoxydans K1	−1.7	0.2	0.98			−73	7				49 ± 4	[21]
Mixed aerobic cultures												
Consortium Pz1-ETBE cultivated:												
Oxic at 30 °C	−0.4	0.3	0.94	90	5	ns (−0.3)	5	0.05	90	5	na	This study
Hypoxic at 30 °C	−0.7	0.2	0.95	89	5	ns (+12)	57	0.9	89	3	na	This study
Oxic at 12 °C	−0.5	0.2	0.98	86	7	ns (+6)	16	0.8	86	4	na	This study
Hypoxic at 12 °C	−0.6	0.1	0.95	85	5	ns (+5)			85	2	na	This study

N: number of data points; na: not applicable; ns: not significant; B%: maximum ETBE biodegradation percentage analyzed for the isotopic composition.
[a] Original data in Rosell et al. [21] was recalculated according to statistics described in Rosell et al. [30].
[b] Results from L108 experiments with growing and resting cells were plotted together.

numbers (2370 *ethB* copies L^{-1}). A qPCR analysis carried out at day 61 showed a dramatic increased in the number of *ethB* gene copies (2.3×10^5 *ethB* copies L^{-1}) which probably reflected the colonization of the groundwater by the relevant microorganism. The level reached # 5×10^6 copies L^{-1} after the third ETBE addition.

By the end of the experiment (# 480 h after the fourth ETBE addition), the number of *ethB* copies returned to the initial level which implies that continuous feeding is a requirement for maintaining the high number of degraders during the biotreatment.

The monitoring of *ethB* gene expression (RT pPCR) that will be carried out, might comfort these results.

4. Conclusions

To the best of our knowledge, this is the first study of a site contaminated by ETBE and the first demonstration of ETBE bioremediation under site conditions. A mixed culture (MC-IFP) composed of *R. wratislaviensis* IFP 2016, *R. aetherivorans* IFP 2017 and *A. tertiaricarbonis* IFP 2003 was chosen for bioremediation rather than the Pz1-ETBE (enrichment on ETBE from indigenous microflorae) to limit TBA accumulation in the aquifer. At the pilot plant-scale, bioaugmentation with MC-IFP and H_2O_2 injection were successful to degrade both ETBE and BTEX. In order to get strong data assessing the bioremediation process both ETBE isotopic fractionation measurements and qPCR on the *ethB* gene present in *R. aetherivorans* IFP 2017 were carried out. The increasing in *ethB* gene copy numbers confirmed that the conditions provided in the pilot plant were suitable for the success of bioaugmentation. The monitoring of *ethB* gene by qPCR along an ETBE bioremediation process might be a good tool testifying of the ETBE biodegradation process. In parallel, a low fractionation was observed in the pilot plant when using MC-IFP, thus corroborating the involvement of the *ethB* gene in the aerobic biodegradation of ETBE.

This study extents the importance of the use of complementary tools such as CSIA and the detection of catabolic genes for a reliable characterization of biodegradation in ETBE contaminated sites and to control the success of bioremediation strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.11.074.

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